Influence of Post-Harvest Storage on the Quality and Stability of *Withania somnifera* (L.) Dunal and *Ocimum sanctum* L.

Synopsis

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Submitted by

Mukta Satsangi

Under the supervision of

Prof. Sayeed Ahmad
(Co-Supervisor)
Coordinator: Centre of Excellence in Unani Medicine (Pharmacognosy and Pharmacology),
In charge: Bioactive Natural Product Laboratory,
School of Pharmaceutical Education and Research,
Jamia Hamdard, New Delhi – 110062

Dr. Akbar Ali
(Supervisor)
Department of Botany, Faculty of Science,
Dayalbagh Educational Institute
Dayalbagh, Agra, Uttar Pradesh – 282005

Department of Botany, Faculty of Science
Dayalbagh Educational Institute (Deemed to be University)
Dayalbagh, Agra – 282005
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Introduction

Medicinal plants are known for their indispensable medicinal properties and have been utilized as folk medicine since the dawn of mankind **(Süntar, 2020)**. The essence of medicinal plants lies in the diverse and wide spectrum of secondary metabolites (active chemical constituents) present in them and synthesised during secondary metabolism **(Seca and Pinto, 2019; Ali *et al.*, 2014)**. These metabolites exhibit biological activities which help in defense and protection mechanism against both abiotic and biotic stress to plants **(Jan *et al.*, 2021; Ashraf *et al.*, 2018)**. Additionally, they possess vast number of beneficial pharmacological and therapeutic activities such as antioxidant, anti-inflammatory, anticancer, antiviral, antibacterial, antifungal, insecticidal, antimalarial, anti-aging, etc. for treating human diseases **(Ćavar Zeljković *et al.*, 2021; Kumar *et al.*, 2021; Ben-Shabat *et al.*, 2020)**. The extensive use of medicinal plants in dietary and food items as nutritional supplements, flavouring agents, and antibacterial agents, which highlights their nutritional relevance, makes them a significant element in nutraceuticals **(Nieto, 2020; Sun *et al.*, 2019)**. Additionally, medicinal plants or their extracts are often used in cosmetology to produce herbal skin care products **(Liyanaarachchi *et al.*, 2018; Ribeiro *et al.*, 2015)**. Therefore, due to multifunctional nature of these bioactive phytoconstituents, they are widely applied in pharmaceutical, nutraceutical, cosmetic industries.

In recent years, the demand of plant-based drugs or products have vigorously spread in the global market **(ALkharashi, 2021; Silva & Pogačnik, 2020).** As a result, the herbal drug and botanical supplement market has expanded internationally **(Gamage *et al.*, 2021).** According to World Health Organisation **(WHO, 2019)**, approximately more than 80% of world's population uses traditional plant-based remedies for the treatment of diseases and wellbeing of mankind. As reported, the global revenue generating from herbal products is expected to reach USD$ 140 billion by 2024 **(Singh *et al.*, 2022; Lo and Shaw, 2019)**. These products are worldwide available and accepted by virtue of their safety and effectiveness **(Do *et al.*, 2019).** But with the growing demand and use, the utmost importance for the consumers is high and safe quality with effective activities of these herbal extracts or products **(Zahiruddin *et al.*, 2023; Jamshidi and Cohen, 2017; Steinhoff, 2019)**.

The quality of the crude plant material (crude drug) is prerequisite to produce any high-quality plant-based drugs or products and to enhance benefits of these products on human health and is mainly

assessed in the terms of bioactive constituents and the efficacy of their corresponding pharmacological activity. These phytonutrients are influenced by pre-harvest factors such as germplasm, plant species, agro-climatic zones, stage of plant collection, harvesting season, etc. (**Li *et al.*, 2020**) and post-harvest processes like drying techniques **(Kroehnke *et al.*, 2018; Antal *et al.*, 2011)**, storage practices **(Rutnik *et al.*, 2022)**, extraction methods, packaging, transportation conditions, or any other kind of processing **(Gião *et al.*, 2013)**.

The industries and market receive surplus crude plant materials (crude drugs) which are not processed immediately into herbal extract, formulation, or product and thus gets collected. During their post-harvest storage, two parameters, storage conditions and duration plays an important role in affecting the composition and efficacy of bioactive constituents **(Sourestani *et al.*, 2014).** The phytoconstituents which are usually present in trace amount, are sensitive in nature and undergo various chemical changes (oxidation, biotransformation, polymerisation, etc.) when exposed to inappropriate storage conditions such as light, temperature, humidity, pH, oxygen **(Mohamadi *et al.*, 2011)**. Such conditions either lead to their degradation or elevation. Even if the crude plant materials are stored under controlled environment but for a prolonged period, changes in phytoconstituents and corresponding biological activities occurs **(Xu *et al.*, 2019)**. Apart from this, distinct changes in organoleptic properties (aroma, taste, texture) also takes place. Thus, post-harvest storage practices exert a great impact on the bioactive constituents and hence on the quality of crude plant materials.

Such changes during post-harvest storage can be reduced or slowed down when stored under appropriate conditions. For this, it is essential to monitor the changes in phytoconstituents of plant material to determine the optimum storage conditions under which these metabolites show best stability and efficacy. Through comprehensive systematic stability studies, quality of the plant material is assessed in diverse environmental conditions to ensure proper storage conditions under which they can be kept without compromising their quality, safety, and efficacy throughout their shelf lives **(Bansal *et al.*, 2016)**. By determining the shelf life of crude plant materials, it can be analysed the loss of phytoconstituents and their activity over time which will allow industries to make informed decisions regarding stored plant material.

The quality aspects of crude plant materials can be determined by chromatographic fingerprinting and

metabolomic profiling **(Amir *et al.*, 2022)**. To analyse the changes in chemical constituents (quantitative and qualitative) in crude plant material when stored under different conditions, analytical techniques such as High-Performance Thin-Layer Chromatography (HPTLC), High-performance liquid chromatography (HPLC), Liquid chromatography–mass spectrometry (LC-MS) or Gas chromatography–mass spectrometry (GC-MS) **(Ahmad *et al.*, 2022)** are used. The chromatogram and digital fingerprint profile/image discloses both qualitative and quantitative aspects of phytoconstituents in any crude plant material in comparison with reference standards **(Khan *et al.*, 2021).**

Literature review

Ample research studies previously have reported the influence of pre-harvest factors on active phytoconstituents content, composition, and activities of medicinal plants. In terms of post-harvest processes, the effect of various drying practices and packaging materials on these phytoconstituents are too reported. But only few studies have reported the impact of storage practices on phytochemicals of raw medicinal plants as discussed below.

Zeljković *et al.* (2021) found that the essential oil (EO) content of *Thymus vulgaris* and *Mentha spicata* decreased about 43% and 19% respectively at end of 25 weeks when stored under 30% humidity and 22-25°C temperature. The % decrease in major constituents of *T. vulgaris* was thymol (50%), carvacrol (60%), p-cymene (56%), γ-terpinene (64%), (*E*)-caryophyllene (86%). The % decrease in major constituents of *M. spicata* was myrcene (29%), limonene (41%), 1,8-cineole (22%), piperitenone oxide (27%), (E)-caryophyllene (63%). The radical scavenging activity of *T. vulgaris* EO decreased about 19% (in 9th week), 25% (in 15th week), 27% (in 25th week) whereas *M. spicata* EO decreased about 24% (in 10th week), 70% (in 15th week), 71% (in 25th week).

The fresh *Asparagus racemosus* (Shatavari) root churna was compared with churnan of two months, six months, and one-year-old sample stored in porcelain jars at ambient temperature by **Rao *et al.* (2021).** The characteristic odor became indistinct after 1 year, the colour changed from creamy to creamy yellow in 6 months and the initial taste (bitter with mild sweeter) became characteristic bitter after 2 months. Saponins decreased from 33.20% to 15.10% after 6 months, and was completely absent after 1 year. Shatavarin was completely absent in 1 year old sample.

Xu *et al.* (2019) investigated the changes in *Camellia sinensis* L. (White tea) aged 1 year, 3 years and 5

years. With prolonged storage duration, decrease was found in total phenolic content (from 19.79% to 17.36%), protein content (from 3.91% to 3.13%), catechins content (from 12.57% to 9.40%) and caffeine content (from 4.27% to 4.15%). While total soluble sugar content increased from 3.62% to 4.17% with duration of storage time. The antioxidant and antidiabetic activity too decreased with increase in storage duration.

Gondaliya *et al.* (2018) reported in seeds of *Cuminum cyminum* L. (cumin), the EO yield decreased from 4.1% to 2.1% and oleoresin yield decreased from 16.11% to 14.63% in 60 months when stored in cotton bags at room temperature. Total phenolic content of methanolic extract reduced from 12.60 to 12.02 mg GAE/g dw up to 36 months and further reduced to 10.08 on storage at and of 60 months. Total flavonoid content showed insignificant change up to 36 months and then reduced to 6.92 mg QE/g dw at end of 60 months. The freshly harvested cumin seed had highest 743 mM AAE/g dw which was gradually decreased to 588 mM AAE/g dw upon storage of cumin seed for 48 months.

Hend *et al.* (2018) reported that in leaves of *Laurus nobilis* L. (Bay laurel) when stored in dry place, the EO % was 1.14% initially which decreased to 0.78%, 0.68% and 0.54% after 4, 8 and 12 months respectively. Major component (1,8 cineol) increased from 56.83% to 61.59% up to 8 months and decreased to 49.3% at 12 months. Oxygen containing monoterpenes increased from 83% to 87.19% up to 8 months and decreased to 77.45% at 12 months. Monoterpene hydrocarbons decreased from 11.48% (0 months) to 9.39, 7.62, and 8.34% after 4, 8 and 12 months of storage respectively. Sesquiterpene-hydrocarbons decreased from 1.61% initially to 1.43%, 0.99% after 4- and 8-months storage and increased to 1.95 % after 12 months. The constituents α-Terpinenyl acetate, sabinene, trans-beta-terpineol and terpinene-4-ol were more stable during storage period.

Ebadi *et al.* (2017) examined the effects of packing methods (packed with air, nitrogen, or under vacuum) and 8 months storage duration on the lemon verbena oil content. With the increase in the storage duration in all packaging methods, citral content was decreased, whereas the amounts of limonene and 1,8-cineole were increased. Packaging of lemon verbena leaves with nitrogen preserved the highest EO content during 8 months of storage and achieved the desired amounts of citral, limonene, and 1,8-cineole.

Griggs *et al.* (2001) collected 19 species of medicinal plants from different regions of Nepal and stored

them in cotton bags at room temperature and reassessed their in vitro biological activity after 6 years. Of the 19 plants, three plants (*Corallodiscus lanuginosus*, *Elephantopus scaber*, and *Sibbaldia micropetala*) completely lost their biological activity. Six plants (*Elsholtzia blanda, Elsholtzia fruticosa*, *Hypericum cordifolium, Hypericum elodeoides*, *Pogostemon enghalensis* and *Valeriana jatamansi*) retained all activity whereas the remaining plants retained only partial antimicrobial activity and light enhanced activity.

Plant profile of selected plants for the study

The two most commonly used medicinal plants, Ashwagandha and Tulsi are selected for our study.

Table 1: Selected plants with their most common part used and activities

Plant species	Part used	Activities
***Ocimum sanctum* L.** (Holy basil, Tulsi) Family: Lamiaceae	Leaves	Anticancer, antioxidant, anti-inflammatory, leishmanicidal, radiation protective, mosquitocidal, antimicrobial, antistress activity, antihyperlipidemic and antidiabetic
***Withania somnifera* (L.) Dunal** (Indian ginseng, Ashwagandha) Family: Solanaceae	Root	Anti-inflammatory, anti-tumor, antimicrobial, immunomodulatory, anti-cancer, anti-diabetic, cardio-protective, neuroprotective

***Ocimum sanctum* L.**

According to system of classification of Arthur Cronquist (1981), taxonomic classification of *Ocimum sanctum* is

Kingdom	Plantae
Division	Tracheophyta
Subdivision	Spermatophytina
Class	Magnoliopsida
Order	Lamiales
Family	Lamiaceae
Genus	*Ocimum*
Species	*sanctum*

Fig 1: *Ocimum sanctum* L. at herbal garden DEI, Agra

Geographical distribution of *Ocimum sanctum*

Ocimum sanctum is considered indigenous to the Indian subcontinent including the Himalayas, Malesia,

and other tropical and subtropical parts of Asia, and is now widely cultivated and naturalized in places around the world, including the Caribbean, Pacific islands, and parts of Africa. Introduction of the species beyond Asia has primarily been for its medicinal and culinary use, as it has been used for these purposes for at least 3000 years. Tulsi is native throughout the world tropics and widespread as a cultivated plant and an escaped weed. The natural habitat of Tulsi varies from sea level to an altitude of 2000 m. It is found growing naturally in moist soil nearly all over the globe (**Wagner *et al.*, 2014**).

Botanical description of *Ocimum sanctum*

Ocimum sanctum is an erect, much branched sub-shrub 30–60 cm tall, with simple opposite green or purple leaves that are strongly scented and hairy stems. Leaves have petiole and are ovate, up to 5 cm long, usually somewhat toothed. Flowers are purplish in elongate racemes in close whorls (**Siva *et al.*, 2016**).

Phytochemistry of *Ocimum sanctum*

Ocimum sanctum leaves are rich in volatile oil (0.7%), phenolics, flavonoids, neolignans, terpenoids and fatty acid derivatives. Caffeic acid, chlorogenic acid, vanillic acid, ocimumnaphthanoic acid and menthylsalicylic glucoside have been isolated from the aerial parts of *O. sanctum*. The presence of commonly occurring phenolic compounds gallic acid, gallic acid methyl ester, gallic acid ethyl ester, protocatechuic acid, 4-hydroxybenzoic acid, vanillin and 4-hydroxybezaldehyde were confirmed by HPLC. Rosmarinic acid, an ester of caffeic acid has been quantified by using APCI mass spectrometry technique. Flavonoids are the major class including methoxy flavonoids and their glycosides (luteolin, isothymusin, cirsimartin), C-glycosides flavonoids (orientin, isoorientin, isovitexin and vicenin) from *O. sanctum*. Compounds such as apigenin, cirsimaritin, salvigenin, crisilineol, eupatorin, isothymusin and gardenin have also been identified and isolated from aerial parts. Eugenol is one of the most distributed phenyls propanoid in the essential oil of *O. sanctum* leaves. Other phenyl propane derivatives such as ociglycoside or eugenyl-β-D-glucoside, citrusin C, ferulaldehyde, bieugenol and dehydrodieugenol were isolated from the leaves of *O. sanctum*. The methanol extract of *Ocimum sanctum* leaves revealed seven novel neolignans named as Tulsinol A to Tulsinol G. These neolignans are formed by the polymerization of eugenol. Three coumarins named ocimarin, aeculetin and aesculin were reported from *O. sanctum* (**Sundaram *et al.*, 2012**).

Traditional and pharmacological uses of *Ocimum sanctum*

Ocimum sanctum has been described as Rasayana drug in the ancient texts of Ayurveda including Charak Samhita, Susrut Samhita, and Rigveda (3500–1600 BCE) to treat cough, respiratory disorders, poisoning, impotence and arthritis. It is considered as one of the sacred plants in India. It is used as a nervine tonic, adaptogen, improving health during cancer and has beneficial effects in stress release. The therapeutic potential of *O. sanctum* has been well documented in Ayurveda and Siddha for healing properties as well as in Greek, Roman and Unani system of medicines for the treatment of skin diseases, common cold, headaches, coughs, malarial fever, diarrhoea, constipation and as an antidote for snake bite. Its leaves have expectorant, carminative, refrigerant, febrifuge, laxative properties, and their infusion is used as a stomachic in gastric disorders of children. Juice of fresh leaves is used as the first-aid remedy for earache. Its seeds are mucilaginous and demulcent and useful in the treatment of genitor-urinary disorders. It can be consumed as herbal tea, decoction (leaves and roots) to treat cough, cold and malarial fever. The paste of green or dried powdered leaves is used to treat ring-worm, skin diseases and vitalizing effect, whereas essential oil as larvicidal. It exhibits various pharmacological activities such as anticancer, antioxidant, anti-inflammatory, leishmanicidal, radiation protective, mosquitocidal, antimicrobial, antistress activity, antihyperlipidemic and antidiabetic activity (**Singh and Chaudhuri, 2018**).

***Withania somnifera* (L.) Dunal**

According to system of classification of Arthur Cronquist (1981), taxonomic classification of *Withania somnifera* is

Kingdom	Plantae
Division	Tracheophyta
Subdivision	Spermatophytina
Class	Magnoliopsida
Order	Solanales
Family	Solanaceae
Genus	*Withania*
Species	*somnifera*

Fig 2: *Withania somnifera* (L.) Dunal at botanical garden DEI Agra

Withania somnifera has been assigned several names such as “Indian ginseng”, “Winter cherry”, “Poison gooseberry” in English, “Punir” or “Asgandh” in Hindi, “Asgand” in Urdu.

Geographical distribution of *Withania somnifera*

It occurs naturally in drier regions extending from the Mediterranean through tropical Africa through South Africa and from the Canary and Cape Verde Islands to the Middle East and Arabia, Afghanistan, Baluchistan, Pakistan, Sri Lanka, China, Nepal, and India. In India, the plant is widely distributed in the drier parts in subtropical regions, especially in Punjab, Haryana, Gujarat, Uttar Pradesh, Maharashtra, Gujarat, and Rajasthan (Nagour) (**Kulkarni, and Dhir, 2008**).

Botanical description of *Withania somnifera*

It is an evergreen, xerophytic, woody, short, tender, perineal shrub that grows about 2 m tall and 1 m wide. Leaves are alternate (opposite to flowering shoots), almost hairless and green at the upper surface and densely hairy underneath. It has dulled, simple, glabrous, green elliptic leaves and small, green, bell-shaped flowers. The fruit is a hairless spherical berry enclosed in an inflated and membranous persistent calyx, 5–8 mm in diameter and orange-red to red when ripe. The fruit has numerous seeds that are pale brown, kidney-shaped, and compressed to a rough, netted surface. Roots are stout, fleshy, tapering, whitish brown bearing fiber-like secondary branches arising from the main root having a strong odour and bitter, acrid taste (**Uddin *et al.*, 2012**).

Phytochemistry of *Withania somnifera*

The biologically active chemical constituents of *W. somnifera* are alkaloids (withanine, withananine, withasomnine, somniferine, somniferinine, somninine, nicotine), steroidal lactones (withanone, withaferin-A, withanolide A, E, F, G, H, I, J, K, L, M), flavonoids (kaempferol, quercetin), salts (cuscohygrine, tropine, pseudotropin, anahygrine, anaferine), steroids (cholesterol, sitoinosides, diosgenin, stigmasterol, β sitosterol, stigmastadien), N containing compounds (withanol, aomnitol, somnisol).

According to reports, more than 12 alkaloids, 40 withanolides, and several sitoindosides were among the numerous constituents found in several parts by different phytochemical analyses. Among this wide spectrum of pharmacologically active principles, only a few are the key players in exhibiting pharmacological attributes

Withanolides are marker compounds as they are characteristically present in Solanaceae members, especially in the *Withania* genus. The dominant is withanolide D and withaferin A.

Among them, withanolides (steroidal lactones) have been used in an increasing number of drug formulations, given their promissory therapeutic abilities. It is reported that withanolide A, withanolide B, 27-hydroxy withanolide B, withanolide D, withaferin A, 16*β*-acetoxy-6*α*, 7*α*-epoxy-5*α*–hydroxy-1-oxowitha-2, 17, 24-trienolide, 5, 7*α*-epoxy-6*α*, 20*α*–dihydroxy-1-oxowitha-2, 24- dienolide along with common steroids, *β*-sitosterol and sitosterol, and their glucosides in *W. somnifera*. New withanolide glycosides from *W. somnifera* roots, named withanoside I to VII, have been isolated, among which class VI is more abundant. Similarly, two chlorinated withanolides, namely, withanolide C and 4-deoxyphysalolactone have been isolated from dried leaves of *W. somnifera* (**Paul *et al.*, 2021; Singh *et al.*, 2011**).

Traditional and pharmacological uses of *Withania somnifera*

It has been used in Indian medicine for a long time. Indian ginseng is famous due to its familiarity with pharmacologic effects and traditional uses of Korean ginseng tea. Designated as the "Sattvic Kapha Rasayana" in Ayurveda, Ashwagandha has been tremendously used as astringent, anti-carbuncle, anti-stress, tonic, narcotic, diuretic and against worms, piles, goitre, leucoderma, nervous breakdown, insomnia and constipation. *Withania* as "Asgand" also finds its mention in the Unani medicine system in Kitab-al-Hashaish written by Dioscorides in 78 A.D. It is the roots of the plant that have been extensively used in Unani and Ayurvedic systems of medicine. Roots are reported to contain 0.13–0.31% alkaloids, volatile oils, glycosides, starch, amino acids reducing sugars, and steroids. Besides, the root, leaves are useful to cure fever and swellings, flowers have been used as astringent, diuretics, fruits are used to treat skin ulcers, tumors, carbuncles and seeds are effective in increasing sperm count as well as in testicular growth (**Dar *et al.*, 2015**). It possesses various pharmacological activities such as anti-inflammatory, anti-tumour, antimicrobial, immunomodulatory, anti-cancer, anti-diabetic, antipyretic, analgesic, cardio-protective, neuroprotective (**Paul *et al.*, 2021**).

Rationale of the study

Most of the information on medicinal plants pertains to chemical constituents and biological activities. Ample studies related to post-harvest physiology are reported on drying methods, extraction methods,

and on packaging materials for medicinal plants. Despite wide application and importance of medicinal plants, there is a dearth of scientific data on the stability and fate of the bioactive constituents in medicinal plants (crude drugs) during their post-harvest storage period. To the best of my knowledge, no study till date has been reported on the shelf life of medicinal plants (crude drugs) and hence no guidelines have been framed till date to determine the shelf life of medicinal plants (crude drugs).

Thus, the right post-harvest storage is an important factor to get a high-quality product. It is important to study the impact of post-harvest storage conditions and duration on the fate of phytochemical constituent's and their content and composition.

This study is aimed to be conducted to assess the effect of different post-harvest storage conditions and duration on the content and composition of phytoconstituents in medicinal plants (crude drugs) by stability studies. Thus, optimum storage conditions will be determined under which bioactive compounds are stable. Also, the study will determine the shelf life of medicinal plants (crude drugs) to maximise the safety and their efficacy to ensure safe and efficacious drugs/products to the consumers.

Objectives

1. Collection, authentication, drying and storage of Ashwagandha and Tulsi and preparation of their extracts/samples
2. To analyze organoleptic, physico-chemical, and phytochemical properties of stored crude materials at different time intervals
3. Development of chromatographic fingerprints and metabolomic profiling of above said plant materials
4. Determination of shelf life on the basis of real time and accelerated stability testing for Ashwagandha and Tulsi samples

Methodology

Methodology for objective 1: Collection, authentication, drying and storage of Ashwagandha and Tulsi and preparation of their extracts/samples

a) Collection and authentication of plant materials

The fresh plant materials of Ashwagandha and Tulsi will be collected. The crude plant material will be authenticated by taxonomist and traditional pharmacopoeias protocol **(API, 2001; API,**

1999).

b) Drying of plant materials

The freshly collected crude plant materials will be dried under shade in air with sunlight protection and controlled airflow till the moisture content reaches below 10% for optimal preservation.

c) Storage of plant materials

The dried crude plant materials will be packed in gunny bags/jute bags. They will be stored under different storage conditions such as cold storage, warehouse, laboratory for 2 years and in stability chamber for 3 months. Sampling will be done after every 3 months (for cold storage, warehouse, laboratory) and every 15 days (for stability chamber) during storage period.

d) Preparation of the extracts/samples

The crude plant materials will be crushed into coarse powder and will be extracted using suitable solvent and method (will be decided at the time of experiment). The dried crude extracts will be transferred into airtight containers and stored at -20°C for further analysis. The extractive yield will be calculated by:

$$\text{Extraction Yield \%} = \frac{\text{Weight of extract}}{\text{Weight of drug (crude plant material) taken}} \times 100$$

Methodology for objective 2: To analyze organoleptic, physico-chemical, and phytochemical properties of stored crude material at different time intervals

a) Analysis of organoleptic properties of stored crude materials at different time intervals

Organoleptic traits such as texture, colour, aroma of the stored crude plant material will be analysed at different intervals by physical assessment.

b) Analysis of physico-chemical properties of stored crude materials at different time intervals

The stored crude plant material will be analyzed for their physicochemical properties such as moisture content, total ash content, acid insoluble ash according to traditional pharmacopoeias protocol given below (**API, Part-I, Volume – IX, 2016**).

Moisture content (loss on drying)

5 to 10 g of powdered drug will be placed in a tared evaporating dish. The drug containing dish will be placed in hot air oven at 105°C for 3 to 5 hours. Drying and weighing at half an hour interval will be continued until difference between two successive weighing corresponds to not more than 0.25%.

$$\% \text{ Loss on drying} = \frac{\text{Loss in weight of sample}}{\text{Weight of drug (crude plant material) taken}} \times 100$$

Total ash content

2 to 3 g of the powdered drug will be incinerated in a tared platinum or silica dish at a carbon, will be cool in a desiccator for 30 min and will be weighed again.

$$\% \text{ Total ash value} = \frac{\text{Weight of total ash}}{\text{Weight of drug (crude plant material) taken}} \times 100$$

Acid insoluble ash

To the crucible containing total ash, dilute hydrochloric acid will be added. Collection of the insoluble matter on an ashless filter paper and washing with hot water until the filtrate is neutral. Transferring the filter paper containing the insoluble matter to the original crucible, drying it on a hot plate and ignite it to constant weight. Allowing the residue to cool in a suitable desiccator for 30 min and weighing again.

$$\% \text{ Acid insoluble ash value} = \frac{\text{Weight of acid insoluble ash}}{\text{Weight of drug (crude plant material) taken}} \times 100$$

c) Analysis of phytochemical properties of stored crude materials at different time intervals

It will be analysed by total phenolic content (TPC) and total flavonoid content (TFC). TPC of stored crude material will be determined by Folin-Ciocalteu method. The absorbance will be measured using a spectrophotometer and gallic acid will be used as a reference standard for the calibration curve. TFC of stored crude drugs will be determined by aluminum chloride method. The absorbance will be measured using a spectrophotometer and rutin or quercetin will be used as a reference standard for the calibration curve (**Parveen *et al.*, 2019**).

Methodology for objective 3: Development of chromatographic fingerprints and metabolomic profiling of above said plant materials

a) Chromatographic fingerprinting of stored crude materials

The fingerprinting of the stored crude plant materials will be performed by High-Performance Thin-Layer Chromatography (HPTLC). The samples of the stored crude plant materials and standards (available in laboratory) will be prepared and separately applied on TLC plates (HPTLC silica gel 60 F254). The plates will be developed using suitable solvent as mobile phase in TLC chamber. The developed plates will be further scanned at suitable wavelengths and selected markers will be quantified (**Zahiruddin *et al.*, 2021**).

b) Metabolomic profiling of stored crude materials

Metabolomic profiling of extracts of stored crude plant materials will be performed by different chromatography techniques High-Performance Thin-Layer Chromatography (HPTLC) or High-performance liquid chromatography (HPLC) or Liquid chromatography–mass spectrometry (LC-MS) or Gas chromatography–mass spectrometry (GC-MS) (**Jan *et al.*, 2022**).

Methodology for objective 4: Determination of shelf life on the basis of real time and accelerated stability testing for Ashwagandha and Tulsi samples

Shelf-life of Ashwagandha and Tulsi samples stored under different conditions will be determined by degradation kinetic studies using Arrhenius equation which describes the relationship between storage temperature and degradation rate. It will be constructed between log K and 1/T (**Parveen *et al.*, 2015**).

The shelf life ($T_{0.9}$) for crude plant material will be determined by:

$T_{0.9} = 0.105/k$, where k is degradation rate constant

The half-life ($T_{0.5}$) for crude plant material will be determined by:

$T_{0.5} = 0.693/k$, where k is degradation rate constant

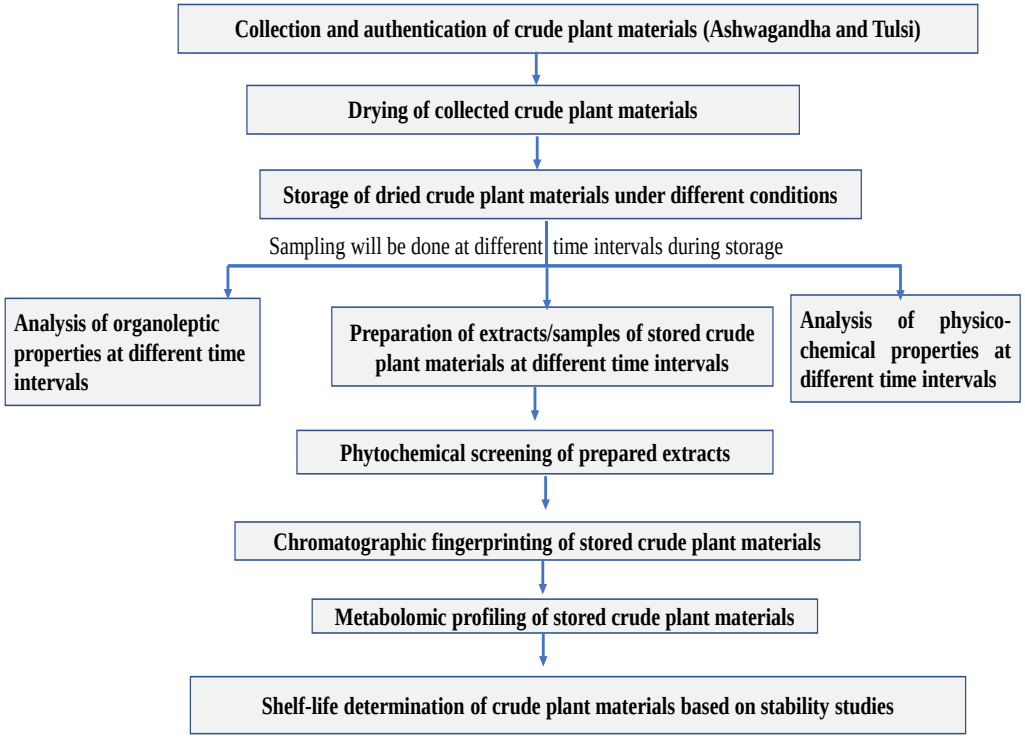
Collection and authentication of crude plant materials (Ashwagandha and Tulsi)
Drying of collected crude plant materials
Storage of dried crude plant materials under different conditions
Sampling will be done at different time intervals during storage
Analysis of organoleptic properties at different time intervals
Preparation of extracts/samples of stored crude plant materials at different time intervals
Analysis of physico-chemical properties at different time intervals
Phytochemical screening of prepared extracts
Chromatographic fingerprinting of stored crude plant materials
Metabolomic profiling of stored crude plant materials
Shelf-life determination of crude plant materials based on stability studies

Fig 3: Plan of work

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